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Short communication

Effects of parasitism on cellular immune response in sheep experimentally infected with *Haemonchus contortus*

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ABSTRACT

This work aimed to study the possible relationships among the magnitude of abomasal worm burden and the proliferation of globular leucocytes and mucosal mast cells in the abomasal mucosa, and the white blood cell count. Eighteen Suffolk × Greyface lambs were infected with *Haemonchus contortus*, and 12 were kept free of nematodes. Blood samples were collected on days 0, 30, and 57 post-infection (p.i.) for leucogram determination. At day 62, all animals were euthanized to count the total number of nematodes recovered in the abomasum and to count the number of mucosal mast cells and globular leucocytes. On day 57, higher levels of parasitism corresponded to lower leucocyte counts. The infected groups had lower lymphocyte counts throughout the experimental period. Animals with higher numbers of parasites had lower neutrophil and eosinophil counts on day 57. The lower the worm burden, the greater the number of mucosal mast cells ($r = -0.85$; $p < 0.01$) and globular leucocytes ($r = -0.87$, $p < 0.01$) observed. The sheep most resistant to haemochosis had greater peripheral and tissue cellular immune responses.

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1. Introduction

The *Haemonchus contortus* parasite is considered the most prevalent parasite throughout the UK and is present in 75–100% of egg per gram (EPG) counts (Mortensen et al., 2003, Waller, 2005). In addition to its high prevalence, *H. contortus* is the most pathogenic nematode found in Brazil (Amarante et al., 2004). The pathogenicity of *H. contortus* is related to the haemorrhage that this nematode causes in the abomasum of infected sheep. Females can lay 5000 eggs on average and cause a loss of blood which can lead to anaemia and hypoproteinaemia of varying degrees, depending on the age of the animal and its degree of immunity (Urquhart, 1998).

Most work on the haematology of animals infected with *H. contortus* is based on EPG of faeces as a determinant of infection. It is known that this parameter may not reflect the actual infection because a high density of parasites in the abomasum makes female worms lay fewer eggs, which could result in a false negative EPG. The degree of infection is also dependent on factors related to climate conditions, such as the rate of contamination of the pasture, as well as grazing behaviour, previous infections, and the physiological state of the animal (Waller, 2005).

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Immunity during gastrointestinal parasite infections involves both humoral and cellular responses (Miller and Horohov, 2006), which are known to be closely dependent. The immune response is dependent on the pattern of gene expression of cytokines induced during infection. Regarding cellular immunity, mast cells and eosinophils are important effector cells against parasitic infections. Nevertheless, the expulsion of nematodes seems to vary with the species of parasite involved. Globular leucocytes (GLs) and mucosal mast cells (MMCs) are found only in animals infected with gastric parasites (Rothwell, 1989), such that GLs are an indicator of the disease. Severe parasitic infections may lead to stress in animals and consequent release of the hormone cortisol into the circulation (Fleming, 1997). This work was designed to study possible relationships among the degree of abomasal worm burden as measured by leucogram, and the proliferation of GLs and MMCs in the abomasal mucosa.

2. Materials and methods

2.1. Animals and experimental diet

This study was conducted on the premises of the Moredun Research Institute in Edinburgh, Scotland. Thirty Suffolk × Greyface lambs (equal numbers of males and females), nine months old and weighing an average of 30 kg were used. The lambs and their mothers were reared in a special shed with no contact with pasture to keep them free of gastrointestinal helminth infection at the beginning of the experiment. The animals were kept in individual metabolic cages during the entire experimental period. The experiment was conducted under UK Home Office Licence with the approval of the Local Ethical Standards Committee.

The diet of the animals was based on barley, urea, and a premix of minerals and vitamins with 85% dry matter and 14.5% crude protein. At the beginning of the experiment, the animals received 750 g of dry matter (DM)/head/day, with the amount offered gradually increased to 1000 g DM/head/day over 15 days. This amount was held fixed until the end of the experiment. Before beginning the experiment, an EPG was performed on each sheep to ensure the absence of parasitic infection.

2.2. Infection and experimental groups

Infective *H. contortus* larvae (L_3), collected from a pure culture of faeces sampled from various sheep infected only by this worm, were used to experimental infection. The faeces were cultured at 20 °C for 10 days, and the infective larvae were recovered using the standard Baermann technique.

Initially, the animals were divided based on the absence or presence of infection (infected, $n = 18$, not infected, $n = 12$). Sheep infection was carried out in series following the protocol recommended by Suttle et al. (1992): 500 infective *H. contortus* larvae (L_3) were administered orally every day, five times a week over six consecutive weeks. Five days after the last blood collection, at 62 days p.i., the lambs were sacrificed (acepromazine 0.1 mg/kg intravenously [IV], thiopental 100 mg/kg IV, followed by the administration of 50 mL of potassium chloride IV) for *post-mortem* analysis. It was verified that the eighteen sheep were equally divided into three groups ($n = 6$) according to the quantity of *Haemonchus* worms in the abomasum recovered during the post-mortem examination. Group A consisted of subjects with a worm count of less than 1000 worms, group B consisted of subjects with a count between 1000 and 5000 worms, and group C was composed of subjects with more than 5000 worms. The 12 non-infected sheep served as controls (group D).

2.3. Blood collection and analysis

On days 0, 30, and 57 after infection, blood samples were collected via jugular venepuncture in Vacutainer® tubes containing sodium EDTA as an anticoagulant. Total and differential leucocytes counts were performed manually by conventional methods (Latimer et al., 2003). Plasma was obtained by centrifugation for 20 min and stored at −20 °C until biochemical analysis. To evaluate potential stress due severe infection, plasma cortisol levels were assessed at day 57 p.i. using a commercial radioimmunoassay kit (Inter Sci Diagnostics, Los Angeles, CA).

2.4. Recovery of H. contortus worms from the abomasum

After euthanasia, the abomasum of the animals was removed. Adult worms were recovered using the technique described by Coop et al. (1986). The contents of the abomasum were collected in a graduated container. The abomasum was washed with a saline solution to remove any adult worms attached to the mucosa. This liquid was also placed in the container. Next, saline solution was added to the material collected to a final volume of 2000 mL. After complete homogenisation, a 10% aliquot (200 mL) was taken, and this aliquot was supplemented with 30 mL of a 5% formaldehyde solution. To calculate the total number of adult worms, the number of helminths was multiplied by 10.

Immature worms were recovered by the technique described by Jackson et al. (1984). The abomasum was opened longitudinally and placed in a plastic container with 2000 mL of saline solution preheated to 37 °C, and the container was incubated at this temperature for three hours. During the incubation period, the container was tightly and was vigorously shaken for 2 min each hour. The collection of the aliquot to be used, the addition of the formaldehyde solution, and the counting of the helminths were completed as described above.

2.5. Processing of the abomasal mucosa

Abomasal tissue samples were fixed at 4 °C for 12 h in a paraformaldehyde/4% PBS (pH 7.4) solution. Next, the samples were transferred to a 10% formalin-saline-calcium solution to be processed in blocks of paraffin wax. To demonstrate the presence GLs and MMCs, sections of the same tissue were stained with carbol chromotrope and toluidine blue, respectively (Huntley et al., 1984). The counting of these cells was performed in 25 fields using

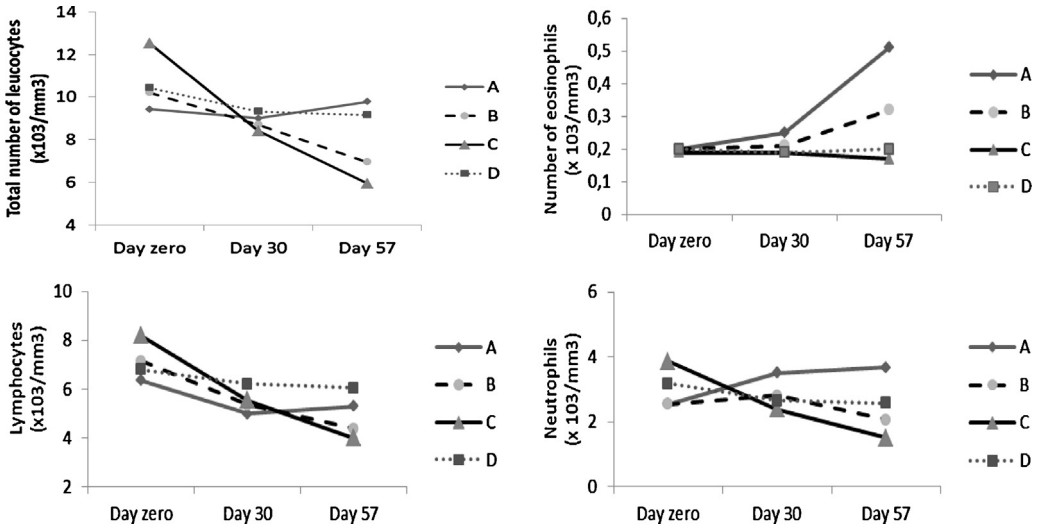

Fig. 1. Influence of worm burden on white blood counts on days 0, 30, and 57 after infection with *Haemonchus contortus*.

an objective lens with 100x magnification. In addition to assessing specific aspects of the staining, the cells were compared with those described by Huntley et al. (1984). The animals were divided, post-mortem, into four groups according to the count of recovered worms in the abomasum. The influence of the number of worms on the haematological parameters and cortisol was studied.

2.6. Statistical analysis

Results were analysed using a computerised statistical programme. The data, which showed a normal distribution, were analysed with a one-way ANOVA and the means were compared with a Duncan test. The number of parasites in the abomasal contents and the EPG were analysed with a nonparametric Kruskal–Wallis test. Linear regression analysis and/or correlation coefficients were used to study the relationships between the variables. The significance obtained in the linear regressions was assessed using an F test. *P*-values lower than 0.05 were considered to be statistically significant.

3. Results and discussion

Fig. 1 shows the white blood counts over all 3 periods for each group (A, B, C and D). Lower total blood leucocyte counts corresponded to greater worm burdens ($r = -0.80$; $p < 0.003$; worm burden = 13.011 – 1.20 leucocyte count), with a clear inverse relationship between parasite load and total blood leucocyte count. This finding indicates that the sheep that were more susceptible to haemochosis, harbouring the greatest worm burden, became leucopaenic during the course of infection. On the 57th day, the sheep from group A showed a number of total blood leucocytes count similar to the control group and the groups kept free of parasites (Leal et al., 2011). Harness et al. (1970) infected calves with three different quantities of *Haemonchus placei* L_3 larvae and noted that the most highly infected animals developed leucopaenia during the seventh and eighth weeks when compared with the pre-infection period.

There is a close relationship between natural resistance and the mechanisms that control the parasite load that allows animals to be productive in spite of low levels of parasitism (Amarante et al., 2004). In a survey conducted by Galina (2011), the author found no difference between the leucograms of sheep infected with *H. contortus* and treated with probiotics and those of sheep only infected with *H. contortus*. Rowe et al. (2008) suggested that there was no modulation of the cellular response to parasitism by *H. contortus*. However, these authors did not examine animals with different degrees of parasitism.

The maintenance of normal total blood leucocyte count in group A was most likely due to a significant increase in the absolute number of neutrophils and eosinophils that offset the mild lymphopaenia. Adams (1993) compared the leucograms of susceptible and non-susceptible sheep prior to infection with *H. contortus* and found that during re-infection, resistant animals showed leucocytosis caused by neutrophilia and accompanied by slight lymphopaenia around the sixth to eighth week of parasitism, i.e., the period of patency. Adams (1993) speculated that at this stage of patency, neutrophil counts could be related to the release of antigens from adult worms in the bloodstream, and that the antigen–antibody complexes formed may lead to greater release of these blood cells.

Group A showed a greater absolute number of eosinophils than did groups B or C. Peripheral eosinophilia during the elimination of parasites has been proposed as a marker of host response against gastrointestinal worms, but this finding does not appear to be universal. Gill (1991) and Adams (1993) found no significant relationship between sheep that were more resistant to *H. contortus* and the number of circulating eosinophils; however, there was a relationship between resistant sheep and the number of eosinophils in the abomasum

The absolute neutrophilia found in this study during the period of patency in the sheep from group A, which

Table 1
Relationship of worm burden to globular leucocytes and mast cell counts in abomasal tissue at 57 day post-infection with *Haemonchus contortus*.

Parasitic group	Median number of cells (25 fields)		
	n	Globular leucocytes	Mucosal mast cells
A ($x < 1000$)	6	53.5	86.0
B ($1000 < x < 5000$)	6	33.5	71.0
C ($x > 5000$)	6	9.0	37.0
P^*	–	0.002	0.003

* Statistical analysis made by Kruskal–Wallis test.

had lower parasite burden, was also observed in other experiments examining *H. contortus* infection (Adams, 1993). Nevertheless, neutrophils are regarded as important effectors of chemotaxic parasiticide and as indicators of an effective host response against helminthosis. Both neutrophils and macrophages are capable of destroying antigens and produce a variety of pro-inflammatory cytokines, which are not usually associated with nematode infections (Galina, 2011).

On day 57 p.i. plasma cortisol concentrations did not differ significantly between the parasitic groups ($p > 0.921$) or between infected and uninfected animals ($p > 0.107$). The mean cortisol concentrations were 32, 48, 24 and 34 nM/L for groups A, B, C and D, respectively. This observation, combined with the relative values of blood components, especially the absence of eosinophilia in the more susceptible group (group C), may indicate that the leucopaenia found in most infected groups can be a result of bone marrow depletion, rather than an effect of cortisol, but since no bone marrow analysis was made, this cannot be proved by the present data. The cortisol results could be influenced by the use of metabolic cages; furthers studies are required under natural conditions to evaluate this hormone with more accuracy.

Table 1 presents the average values of the GLs and MMCs. The lower the worm burden (WB), the greater the MMCs observed ($r = -0.85$; $p < 0.01$; MMCs = 86.8 – 0.00588 WB). Lower WB values were also associated with greater numbers of GLs ($r = -0.87$, $p < 0.01$; GLs = 47.6 – 0.00376 WB). Eosinophils and neutrophils are derived from bone marrow, have short lives, and have phagocytic and secretory activities against aggressive agents. Neutrophil production is regulated by (among other factors) T cells, and neutrophils are attracted to the gastrointestinal mucosa by chemotactic factors released by mast cells (Balic et al., 2002). As gastrointestinal mast cells are modulated by T cells, it is possible that there is a relationship between eosinophils and neutrophils in the bloodstream and the number of gastrointestinal mast cells, which are fundamental to the expulsion of parasites. Thus, neutrophilia and peripheral eosinophilia might reflect the number of tissue mast cells (Gill et al., 1994). In our work, neutrophils, circulating eosinophils, and mast cells in the abomasal tissue were more abundant in the groups that had lower worm burdens, which could indicate that these sheep were more resistant to parasitism. The lower the parasite count, the higher the number of intraepithelial and mucosal mast cells in the abomasal tissue. These results confirm those previously described by Williamson et al. (1994). In a study by Shakya et al. (2009), the authors found that sheep naturally resistant to parasites (low parasite load) displayed significant increases in eosinophils, GLs and MMCs.

Both cellular immunity and humoral immunity are parts of the adaptive response of mammals and are actively involved in nematode infections (Miller and Horohov, 2006). The effects of nematode infection are closely related to the nutritional status of the host. It is known that well-fed animals may respond better to infection than those subjected to an inadequate diet (Bricarello et al., 2005), which often occurs in breeding. Nematodes interfere with the ability of the host to utilise nutrients efficiently (Miller and Horohov, 2006), reducing the cellular immune response of these animals against parasitism. In our study, sheep that had lower worm burdens after experimental infection had better peripheral and tissue cellular immune responses, making them able to coexist in equilibrium with parasitism by *H. contortus*.

Conflict of interest statement

The authors have no competing interests.

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